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Behavior of Different Shiga Toxin-Producing *Escherichia coli* Serotypes in Various Experimentally Contaminated Raw-Milk Cheeses

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Shiga toxin-producing *Escherichia coli* (STEC) is an important cause of food-borne illness. The public health implication of the presence of STEC in dairy products remains unclear. Knowledge of STEC behavior in cheeses would help to evaluate the human health risk. The aim of our study was to observe the growth and survival of experimentally inoculated STEC strains in raw-milk cheeses manufactured and ripened according to five technological schemes: blue-type cheese, uncooked pressed cheese with long ripening and with short ripening steps, cooked cheese, and lactic cheese. Cheeses were contaminated with different STEC serotypes (O157:H7, O26:H11, O103:H2, and O145:H28) at the milk preparation stage. STEC growth and survival were monitored on selective media during the entire manufacturing process. STEC grew (2 to 3 $\log_{10}$ CFU $\cdot$ g^{-1}) in blue-type cheese and the two uncooked pressed cheeses during the first 24 h of cheese making. Then, STEC levels progressively decreased in cheeses that were ripened for more than 6 months. In cooked cheese and in lactic cheese with a long acidic coagulation step (pH < 4.5), STEC did not grow. Their levels decreased after the cooking step in the cooked cheese and after the coagulation step in the lactic cheese, but STEC was still detectable at the end of ripening and storage. A serotype effect was found: in all cheeses studied, serotype O157:H7 grew less strongly and was less persistent than the others serotypes. This study improves knowledge of the behavior of different STEC serotypes in various raw-milk cheeses.

Shiga toxin-producing *Escherichia coli* (STEC) is recognized as an important cause of food-borne illness. Pathogenic STEC (also called enterohemorrhagic *E. coli* [EHEC]) is associated with severe human disease such as bloody diarrhea and in some dramatic cases hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (1, 2). Human infection is typically acquired through the ingestion of contaminated food (raw ground beef, dairy products, vegetables, etc.) or water (3–8).

Escherichia coli O157:H7 was the first STEC serotype isolated in the early 1980s and is implicated in the majority of the outbreaks and HUS cases (6, 9). However, other serotypes, such as *E. coli* O26:H11, *E. coli* O103:H2, *E. coli* O145:H28, and *E. coli* O111:H8, have also been implicated in outbreaks (10).

Cattle are the main animal reservoir of STEC (6). Infected cattle can carry the bacteria in their gastrointestinal tract without any symptoms of disease (11, 12) and shed them in their feces (13). However, these pathogens have also been isolated from the feces of other species of domestic animals, including goats and sheep (14). These animals seem to carry *E. coli* serogroups different from those carried by cattle (O26, O91, O115, O128, and O130) (15). Virulence genes or STEC strains have been found in raw milk and raw-milk cheeses (16–18). Detailed investigations have shown that milk can become contaminated during milking or processing if hygiene rules are not followed (19). Once milk has been contaminated with STEC, the organisms can be present in milk products. Cheeses have been characterized as safe food by some authors (19). Nevertheless, outbreaks involving cheeses have been reported worldwide (3, 16, 20–22). The presence of pathogenic bacteria, including STEC, in cheeses is a major concern for food safety authorities. To address the problem, the U.S. Food and Drug Administration requires a minimum of 60 days' aging for cheeses made from unpasteurized milk (23, 24).

To date, most authors have focused their work on the behavior of *E. coli* O157:H7 during cheese manufacturing (23–29), but little is known about the behavior of other STEC serotypes.

The aim of this study was to extend knowledge of the behavior of O157:H7 and non-O157:H7 STEC strains in different types of cheese. To this end, the behavior of different STEC serotypes was evaluated during cheese making (0 to 72 h, depending on the cheese type) and the ripening and storage of cheeses prepared according to the methods for the main five cheese types: blue-veined cheese (raw sheep's milk), lactic cheese (LC; raw goat's milk), cooked cheese (CC; raw cow's milk), and two uncooked pressed cheeses (UPCs; raw cow's milk).

MATERIALS AND METHODS

Bacterial strains. Eight different strains were used in the present study (Table 1): three *E. coli* O26:H11, two *E. coli* O103:H2, one *E. coli* O145:H28, and two *E. coli* O157:H7 strains. The serotypes tested were chosen according to their pathogenicity (classification of verotoxigenic *E. coli* serotypes into the seropathotypes described by Karmali et al. [35]) and their prevalence in dairy products (16, 36). They were all isolated from milk products and carried the virulence genes *eae* and either stx_1 or stx_2 (30, 32). All strains were kept in cryobeads (AEB 400100; AES Laboratories, Combourg, France) and maintained at −80°C.

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TABLE 1 Strains used in this study[a]

Serotype	Name (origin)[b]	Virulence genes	Cheese tested				
			BTC	UPC1-LR	UPC2-SR	CC	LC
O26[c]:H11[d]	7501A (raw cow's milk cheese)	*eae*,[e] stx_1[c]		X[f]	X[f]		X[f]
	L23A (sheep's milk)	*eae*, stx_1	X[f]		X[f]	X[g]	X[f]
	F15338 (raw cow's milk cheese)	*eae*, stx_1			X[f]		X[f]
O103[h]:H2[c]	F73120 (raw cow's milk cheese)	*eae*, *stx*, stx_1			X[f]		X[f]
	F12983a (sheep's milk)	*eae*, *stx*, stx_1	X[f]		X[f]		X[f]
O145[c]:H28[d]	F324 (raw cow's milk cheese)	*eae*, stx_2			X[f]		X[f]
O157:H7[i]	721.4 (sheep's milk)	*eae*, stx_2	X[f]	X[f]	X[f]	X[g]	X[f]
	6222-16.3 (raw goat's milk cheese)	*eae*, stx_2			X[f]		X[f]

[a] BTC, blue type cheese; UPC1-LR, uncooked pressed cheese with long ripening step; UPC2-SR, uncooked pressed cheese with short ripening step; CC, cooked cheese; LC, lactic cheese.
[b] All the strains used were obtained from the French National Reference Laboratory of STEC.
[c] Confirmed by the protocol described by Perelle et al. (30).
[d] Confirmed by the protocol described by Madic et al. (31).
[e] Confirmed by the protocol described by Nielsen et al. (32).
[f] $n = 3$.
[g] $n = 2$.
[h] Confirmed by the protocol described by Perelle et al. (33).
[i] Confirmed by the protocol described by Miszczycha et al. (34).

Inocula. Each strain was revived in 10 ml brain heart infusion (BHI; bioMérieux, Marcy l'Etoile, France) by incubation at 37°C for 24 h. Then, 100 μl was transferred into 10 ml of buffered peptone water (BPW; bioMérieux), incubated at 37°C for 24 h, and stored at 8°C for 48 h in order to simulate the storage conditions of milk and to cold adapt the strains. After turbidity measurements (Densimat photometer; bioMérieux), the cold-adapted strains were diluted in order to inoculate the raw milk during the maturation step, before renneting at 10^2 CFU · ml^{-1} (Fig. 1). The inoculation level was checked by plating inocula on plate count agar (PCA; bioMérieux). Colonies were enumerated after 24 h of incubation at 37°C.

All the strains were tested with the smallest cheeses with a short ripening step (uncooked pressed cheese with a short ripening step and lactic cheese). Only a small number of strains were tested in blue-type cheese (BTC), uncooked pressed cheese with a long ripening step, and cooked cheese, which involve more complicated logistics (according to the size and weight of the cheeses and because of storage during ripening for >4 months). More precisely, *E. coli* O157:H7 and O26:H11 were tested because of their implication in cheese outbreaks (3, 16, 37). Different strains of serotypes O103:H2 and O145:H28 were also tested because of their pathogenicity and the lack of information about their behavior in raw-milk cheeses. Strains from the lab collection were selected according to their origin and the type of cheese; e.g., for cheese made with sheep's milk, only STEC strains isolated from sheep dairy products were chosen. There was an exception for serotype O157:H7 because owing to its low prevalence in cow's milk dairy products, no *E. coli* O157:H7 isolate from such a source was available in our collection. All strains were separately inoculated into raw milk before the coagulation step, in triplicate (except for the cooked cheese technology, where $n = 2$) (Table 1).

Cheese making. Cheeses were made in an experimental cheese dairy facility at the Unité de Recherches Fromagères (URF; INRA) in Aurillac, France, using five different cheese-making schemes. Five different cheeses were prepared to be representative of the main cheese technology schemes defined by Lenoir et al. (38). Then, blue-veined cheese (BTC) with raw sheep's milk, CC, UPC (with a long ripening step [UPC1-LR] or with a short ripening step [UPC2-SR]) with raw cow's milk, and LC with raw goat's milk were prepared. The main steps of the process distinguishing them are summarized in Fig. 1. The details of cheese making were previously described in a review of cheese-making technology (39, 40).

For each trial, cheeses were checked for the absence of *E. coli* O26:H11, O103:H2, O145:H28, or O157:H7 in raw milk.

Physicochemical analysis of cheese. The different analysis points at the different stages of the cheese making and ripening were chosen according to the cheese-making schemes and the advice of cheese producers. The pH was measured directly in the core of the cheese. For the surfaces (when possible), the rind was homogenized before the analysis. pH was measured with a 926 VTV pH meter with an Ingold 406 MX electrode (Mettler-Toledo S.A., Viroflay, France). The values of water activity (a_w) in all cheeses were measured by a LabMaster-aw apparatus (Novasina, Lachen, Switzerland). Lactate content was determined by the enzymatic method at the LARF laboratory (LARF-Actilait, Mamirolle, France).

Sampling, counting, and detection of STEC strains. Samples for enumerating STEC strains were taken from milk and from cheese in cores and rinds (only for the two UPCs and CC for the rinds) during different stages of cheese making, ripening, and storage, depending on the cheese-making scheme tested. These analysis points represented potential steps which could have an impact on STEC behavior (because of pH, temperature, etc.). Aseptically collected samples of 50 ml milk or 50 g cheese were diluted (1:5) in sterile BPW and homogenized for 60 s in a stomacher (AES Laboratories). Further decimal dilutions were performed with sterile tryptone salt (TS; bioMérieux) and spread onto selective agar.

For enumeration of serogroup O157 strains, 100 μl of each sample was plated onto specific O157:H7 ID medium (bioMérieux) and SMAC medium (Biokar, Beauvais, France) to which 0.05 mg · $liter^{-1}$ of cefixime and 2.5 mg · $liter^{-1}$ of tellurite were added in order to inhibit other flora. The same medium was used without antibiotic in order to facilitate the isolation of stressed bacteria.

For enumeration of serogroups O26, O103, and O145, 100 μl of each sample was plated onto specific coli ID medium (bioMérieux) and the specific medium described by Possé et al. (41).

All enumerations were performed in triplicate. After incubation for 24 h at 37°C, presumptive bacteria were tested by reverse transcription-PCR (RT-PCR) as described below.

When a strain in a sample could not be enumerated, an enrichment step was performed and STEC strain detection was performed to check whether the target strain was still present. More precisely, the remaining enrichment broth was supplemented with acriflavine and incubated for 24 h at 37°C for serogroups O103 and O145 and 24 h at 41.5°C for serogroups O26 and O157 (42). Immunomagnetic separation (IMS) using beads coated with specific antibodies against the serogroup of the target strain was performed with Dynabeads (Invitrogen, Cergy Pontoise, France). IMS was performed according to the manufacturer's instructions. The resulting IMS solutions were plated onto a selective differential agar medium as described above. All enrichment broths were also plated

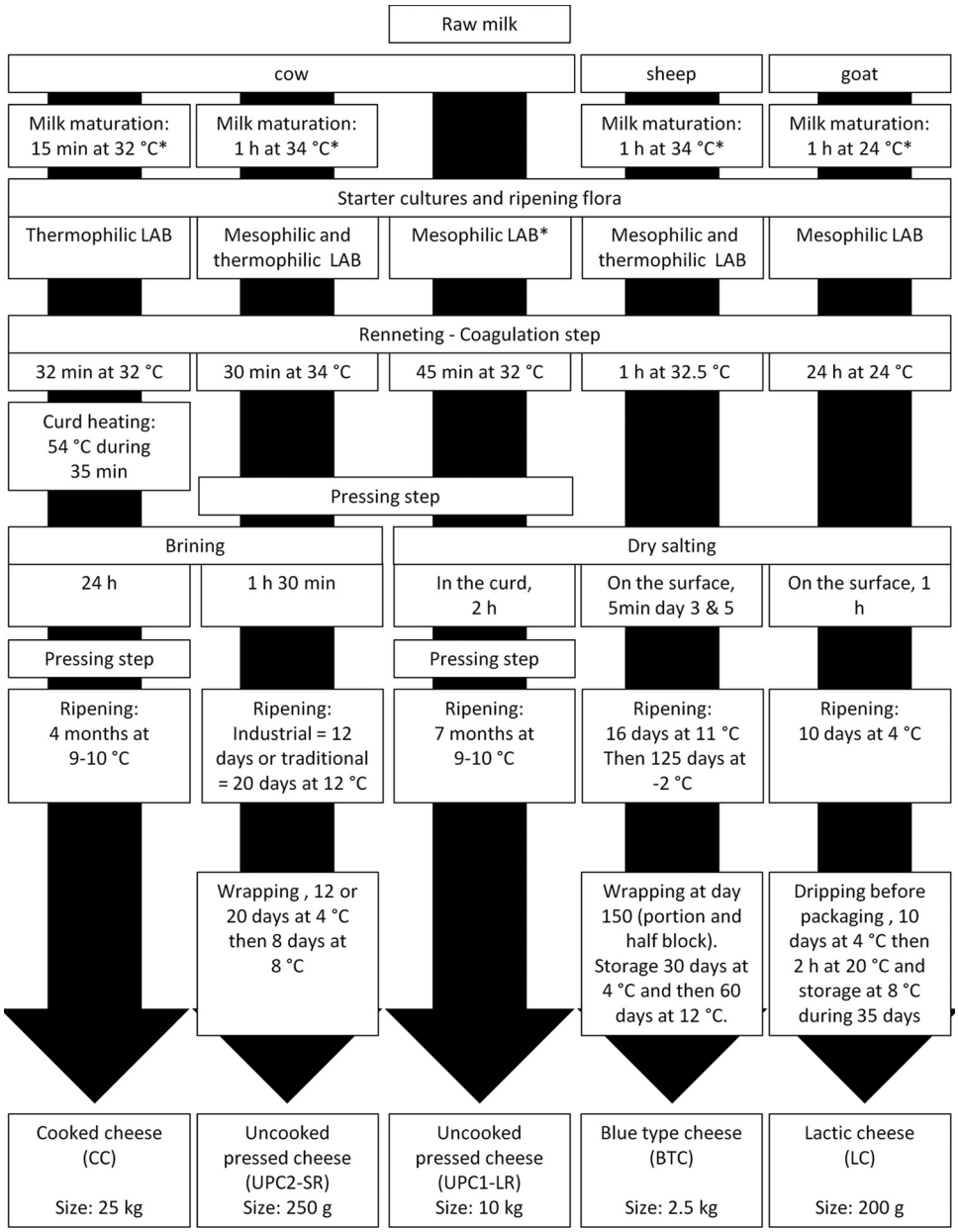

FIG 1 Flow diagram of the characteristics of the different cheese-making schemes tested. LAB, lactic acid bacteria; *, inoculation of STEC.

directly onto the selective differential agars. After 24 h of incubation at 37°C, presumptive colonies were tested by RT-PCR as described below.

Confirmation and characterization of the target bacteria by real-time PCR. For DNA extraction, a single colony was boiled in DNA- and DNase-free water for 15 min and then centrifuged for 3 min at 13,000 × *g*. The supernatant containing the DNA was stored at −20°C until used.

Determination of the virulence genes *eae* and stx_1 or stx_2 was performed using the protocols described by Nielsen et al. (32) and Perelle et al. (30). Confirmation of the presence of *E. coli* O157:H7, the O antigens of *E. coli* O26, O103, and O145, and the flagellar H2, H11, and H28 antigen genes was performed using the protocols described by Miszczycha et al. (34), Perelle et al. (30, 33), and Madic et al. (31), respectively. Each reaction mixture (30 μl) comprised 1× TaqMan gene expression master mix (Applied Biosystems, Foster City, CA), primers and probes concentrated according to the authors' indications, 1× IPC mix and IPC DNA (TaqMan exogenous internal positive control reagents; Applied Biosystems), molecular-grade H_2O, and 3 μl of template DNA.

Thermal cycling and detection were performed using a StepOne Plus system (Applied Biosystems). The generation of fluorescence for each sample was monitored at the end of each elongation step. A fluorescent signal 10 times higher than the standard deviation (SD) of the mean baseline emission was indicative of a positive detection. The threshold cycle was defined as the PCR cycle at which the fluorescence intensity rose above the baseline (30).

Statistical analysis. To study the effect of the strain (in LC and UPC2-SR), serotype (in BTC, CC, LC, and the two UPCs), type of ripening (in UPC2-SR), and storage mode (in BTC) on the behavior of STEC throughout the manufacturing process, a linear model describing the amount of STEC on a decimal logarithmic scale as a function of two dependent variables, time of measurement and the effect studied, was used. Each

model was therefore written $Y = X\beta + \varepsilon$, where Y is the independent variable and represents the vector of observed data (counts) on the log scale, X is the dependent variable studied (strains, serotype, time of ripening, or storage mode), β is an unknown vector of fixed-effects parameters, and ε is an unknown random-error vector modeling the statistical noise around $X\beta$.

As measurements were repeated at irregular intervals, a continuous-time model was needed to describe the covariance among the errors. The spatial power covariance structure in the Mixed procedure of SAS (version 9.2) was used.

For each technology, when different strains of the same serotype were used, we first tested the strain effect by serotype. If the strain factor had no significant effect, then the curves were used to test the serotype effect. Additionally, the impacts of the ripening time and the storage conditions were tested for UCPC2-SR (traditional versus industrial ripening) and for BTC (two different storage conditions), respectively. The fixed effect was considered significant when the P value computed by the F test was less than 5%.

RESULTS

BTC (sheep's milk). (i) *E. coli* O157:H7, O26:H11, and O103:H2 behavior. Only one strain each of *E. coli* O26:H11, *E. coli* O103:H2, and *E. coli* O157:H7 was tested in triplicate for BTC (Table 1).

In the cheese core, the level of STEC increased by 1 $\log_{10}$ CFU · g^{-1} for the O157:H7 strain during the first 24 h of manufacture, whereas the *E. coli* O26:H11 and O103:H2 strain concentrations increased by 3 $\log_{10}$ CFU · g^{-1} and 2 $\log_{10}$ CFU · g^{-1}, respectively (Fig. 2). Seven days after curding, the concentration of the *E. coli* O157:H7 strain decreased rapidly and remained detectable only after enrichment, but at 240 days it could not be isolated even after enrichment. The level of *E. coli* O26:H11 decreased during ripening and was detected only after enrichment at 240 days. The level of *E. coli* O103:H2 decreased during ripening, and at day 240, the strain was undetectable even after enrichment. Whatever the storage mode, the growth of the *E. coli* O26:H11 strain was significantly higher, and this serotype was more persistent than the other serotypes ($P < 0.002$).

Whatever the serotype, the storage mode of BTC had no significant effect on STEC behavior ($P > 0.9$ for the three serotypes).

(ii) Physicochemical characteristics. Whatever the STEC serotype inoculated, the pH in the cheese cores decreased from 6.6 to 4.91 during the first 3 days and increased until day 90, reaching 6.69, and then decreased again (Fig. 3). Lactate content was 1,179 mg · 100 g^{-1} (SD = 41 mg · 100 g^{-1}) at 4 days and decreased during ripening; at day 150, it was only 638 mg · 100 g^{-1} (SD = 209 mg · 100 g^{-1}). The a_w in the cheese core gradually decreased to reach 0.898 (SD = 0.006) at day 240.

UPC1-LR (cow's milk). (i) *E. coli* O157:H7 and O26:H11 behavior. Only one strain each of *E. coli* O26:H11 and *E. coli* O157:H7 was tested in triplicate for UPC1-LR (Table 1).

For UPC1-LR, the concentration of the *E. coli* O26:H11 strain reached 6 $\log_{10}$ CFU · g^{-1} during the first 24 h of cheese making, whereas the concentration of the *E. coli* O157:H7 strain reached only 4 $\log_{10}$ CFU · g^{-1} (Fig. 2). The STEC concentration remained constant between day 1 and day 60. Their levels declined during ripening and after day 210 dropped below the enumeration limit for the *E. coli* O157:H7 strain in the rind and core and for the *E. coli* O26:H11 strain in the rind. *E. coli* O26:H11 levels decreased more slowly in the core than in the rind and were still at a concentration of 3 $\log_{10}$ CFU · g^{-1} at day 240. The level of *E. coli* O26:H11 was significantly higher than that of *E. coli* O157:H7 both in the core ($P < 0.0001$) and in the rind ($P = 0.0018$).

(ii) Physicochemical characteristics. Whatever the STEC serotype inoculated, the cheese core pH decreased from 6.79 to 5.19 during the first day and then increased slowly in the core to reach 5.52 at day 240 (Fig. 3). In the rind, the pH increased slowly from 7.34 at day 60 to 7.64 at day 240. Lactate content remained stable at about 1,145 mg · 100 g^{-1} (SD = 80 mg · 100 g^{-1}) during ripening.

In the core of the cheese, a_w decreased slowly to reach 0.943 (SD = 0.002) at day 240. In the rind, a_w decreased from 0.941 (SD = 0.001) at day 60 to 0.922 (SD = 0.001) at day 240.

UPC2-SR (cow's milk). (i) *E. coli* O157:H7, O26:H11, O103:H2, and O145:H28 behavior. Three strains of *E. coli* O26:H11, two strains of *E. coli* O103:H2, one strain of *E. coli* O145:H28, and two strains of *E. coli* O157:H7 were tested in triplicate (Table 1).

As observed with UPC1-LR, the concentrations of all four serotypes increased during the first 24 h of manufacturing (Fig. 2). At day 1, the levels of all strains of *E. coli* O103:H2 and *E. coli* O145:H8 reached 5 $\log_{10}$ CFU · g^{-1}. The levels of all the *E. coli* O26:H11 strains increased to reach 4 to 5 $\log_{10}$ CFU · g^{-1}. The level of the *E. coli* O157:H7 strains reached 3.3 $\log_{10}$ CFU · g^{-1}. During ripening and storage, the concentrations of the different STEC strains remained constant until the end, at day 40. For the same serotype, there was no significant difference between the strains tested. Whatever the serotype, no significant difference in the levels in the core and the rind ($P > 0.46$ for the core and the rind) was observed between traditional (which lasts 20 days) and industrial (which lasts 12 days) ripening conditions. Whatever the ripening conditions (traditional or industrial), the levels of serotypes O103:H2 and O145:H28 were significantly similar in the core ($P = 0.915$ for traditional ripening; $P = 0.365$ for industrial ripening) and in the rind ($P = 0.43$ for traditional and industrial ripening). The levels of serotypes O26:H11 and O145:H28 were also significantly the same in the rind ($P = 0.22$ for traditional ripening; $P = 0.07$ for industrial ripening).

The level of serotype O26:H11 was significantly lower than that of O145:H28 in the core and that of serotype O103:H2 in the core and in the rind ($P < 0.05$). The level of serotype O157:H7 was significantly lower than the levels of serotypes O26:H11, O103:H2, and O145:H28 in the core and in the rind ($P < 0.05$).

(ii) Physicochemical characteristics. Whatever the STEC serotype inoculated, the cheese core pH decreased from 6.6 to 5.3 during the first day. It increased slowly after day 5 to reach 5.80 at day 40 (Fig. 3). Lactate content at day 20, 1,109 mg · 100 g^{-1} (SD = 61 mg · 100 g^{-1}), was higher than that at day 5, 996 mg · 100 g^{-1} (SD = 152 mg · 100 g^{-1}), and that at day 32, 649 mg · 100 g^{-1} (SD = 88 mg · 100 g^{-1}).

The a_w in the cheese core remained constant, with a value of 0.974 (SD = 0.0023) at day 40. In the rind, a_w remained constant with a value of 0.980 (SD = 0.0033) at day 40.

CC (cow's milk). (i) *E. coli* O157:H7 and O26:H11 behavior. Only one strain each of *E. coli* O26:H11 and *E. coli* O157:H7 was tested in duplicate (Table 1).

During the first hours of cheese making, no growth was observed in any of the STEC serotypes (Fig. 2): no *E. coli* O26:H11 or *E. coli* O157:H7 was enumerated at 1.75 h after their inoculation. Then, throughout the ripening for both the core and the rind, strains could be isolated only after enrichment. There was no serotype effect for the CC technology ($P = 0.249$ for the core; $P =$

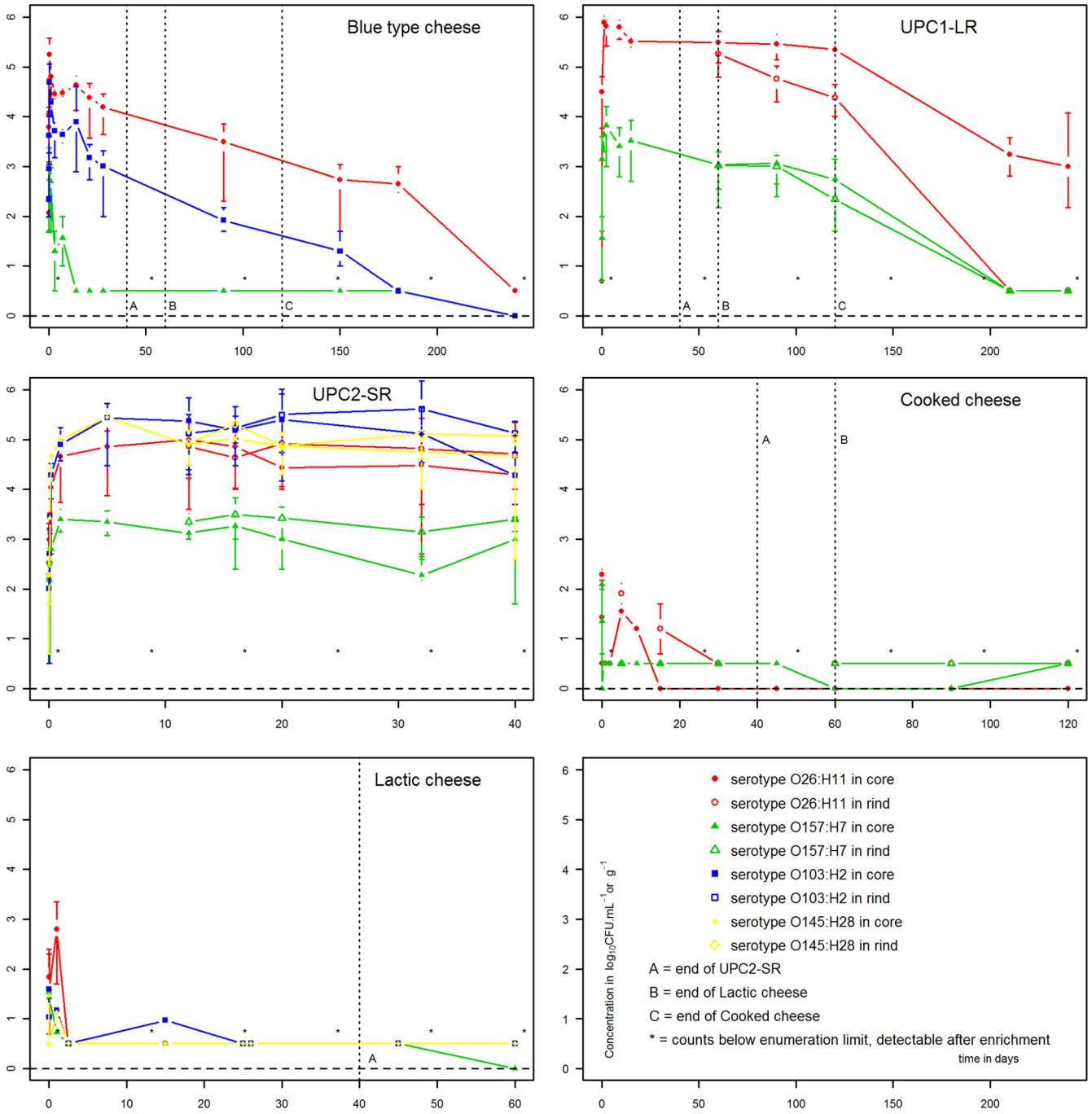

FIG 2 Average counts (*y* axis, in $\log_{10}$ numbers of CFU per milliliter or per gram) of the different STEC serotypes during the manufacture of the different cheeses tested. The end of the shorter technologies is indicated in order to visualize the impact of time on STEC behavior. Vertical bars represent minimal and maximal enumeration values.

0.349 for the rind). In the core, at day 120, the *E. coli* O157:H7 strain was isolated in only one of the two replicates after enrichment. No *E. coli* O26:H11 strains were isolated. In the rind, strains were isolated after enrichment for both replicates of the O26:H11 strain and for only one replicate of the O157:H7 strain.

(ii) Physicochemical characteristics. Whatever the STEC serotype inoculated, during the first hours of cheese making, the pH decreased from 6.6 to 5.38 in the cheese core and remained stable for 1 month. It then increased slowly, reaching 5.82 at day 120. Lactate content increased from 665 mg · 100 g^{-1} (SD = 65 mg · 100 g^{-1}) at 22 h to 1,329 mg · 100 g^{-1} (SD = 115 mg · 100 g^{-1}) at day 45 and then decreased to 1,034 mg · 100 g^{-1} (SD = 64 mg · 100 g^{-1}) at day 120.

In the cheese core, a_w remained stable between day 5 and day 15 and then decreased, reaching 0.975 (SD = 0.0027) at day 120. In the rind, a_w increased from 0.974 (SD = 0.0035) to 0.980 (SD =

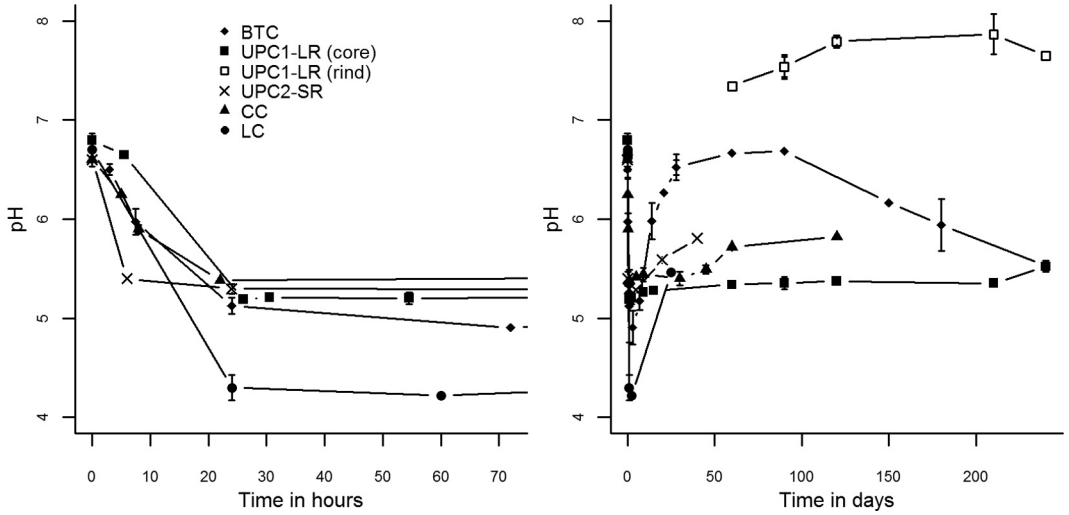

FIG 3 Evolution of the pH values (average counts) during the first hours of cheese making and during the ripening and storage of the cheeses. CC, cooked cheese.

0.0025) between day 15 and day 45 and then decreased to 0.964 (SD = 0.0038) at day 120.

LC (goat's milk). (i) *E. coli* O157:H7, O26:H11, O103:H2, and O145:H28 behavior. Three strains of *E. coli* O26:H11, two strains of *E. coli* O103:H2, one strain of *E. coli* O145:H28, and two strains of *E. coli* O157:H7 were tested in triplicate (Table 1).

During the first hours of cheese making the levels of serotypes O103:H2, O145:H28, and O157:H7 were the same as those in milk (Fig. 2). The concentrations of the three *E. coli* O26:H11 strains increased by only 1 $\log_{10}$ CFU · g^{-1} and decreased until the end of the coagulation step (24 h). As observed for BTC and UPC1-LR, the increase in the serotype O26:H11 count was significantly higher than that that for the other serotypes: $P = 0.0054$ for O26:H11 versus O103:H2, $P < 0.0001$ for O26:H11 versus O157:H7, and $P = 0.0126$ for O26:H11 versus O145:H28. In contrast, there was no difference between serotypes O103:H2, O145:H28, and O157:H7, in pairs. The behavior of strains belonging to the same serotype was significantly similar. At the end of the demolding step (60 h), no strains were enumerable, though they could still be detected after enrichment. During ripening and storage, STEC levels remained detectable. Nevertheless, at day 60, four strains (one *E. coli* O26:H11 strain [strain 7501A], one *E. coli* O103:H2 strain [strain F73120], and the two *E. coli* O157:H7 strains) could not be isolated even after enrichment, whereas the other strains remained present.

(ii) Physicochemical characteristics. Whatever the STEC serotype inoculated, the pH decreased dramatically to reach 4.21 at day 2 and then increased slowly to reach 5.26 at day 25 (Fig. 3). Lactate content decreased from 801 mg · 100 g^{-1} (SD = 46 mg · 100 g^{-1}) at day 2 to 343 mg · 100 g^{-1} (SD = 122 mg · 100 g^{-1}) at day 25.

a_w decreased in the core of the cheese from 0.994 (SD = 0.001) at day 2 to 0.967 (SD = 0.0038) at day 45.

DISCUSSION

In this study, the fate of four STEC serotypes was examined during cheese making and the ripening and storage of raw-milk cheeses prepared according to five different schemes. Currently, *E. coli* O157:H7 is the most studied serotype and the most often implicated in outbreaks of STEC illness from dairy products. To our knowledge, this is the first time that the behavior of serotypes O26:H11, O103:H2, and O145:H28 has been monitored during cheese manufacturing and compared with the behavior of serotype O157:H7. The behavior of STEC during cheese making and ripening varied according to the cheese-making schemes. Each cheese type has its own physicochemical parameters (pH, temperature, a_w) that can affect the growth or the survival of STEC.

Factors that inhibit STEC growth. Our results showed that two physicochemical factors seem to inhibit the growth of the STEC during the first hours of cheese making. The first was sudden, rapid acidification. In the case of LC, the coagulation step lasted 24 h at 18°C with a rapid decrease in pH (under 4.3). *E. coli* is not able to grow at this pH value (43). After this step, STEC remained detectable only after enrichment. The findings of an earlier study led by Vernozy-Rozand et al. (44) confirm our results. They observed that *E. coli* O157:H7 artificially inoculated into raw goat's milk at 2 $\log_{10}$ CFU · ml^{-1} decreased during the first 24 h of goat's milk acid cheese making (pH 4.3). Their results showed that after removal from the mold, STEC was recovered only after enrichment. They isolated STEC after enrichment in their lactic cheese even after 42 days of ripening, which is in agreement with our results.

The second factor influencing STEC growth is high temperature. In CC, after heating the curd at 54°C for 35 min (1 h after STEC inoculation in the milk and before pressing) (45), STEC was isolated only after enrichment. Different studies have shown that *D* values (decimal reduction time) are strain, serotype, and food matrix dependent. Authors have observed *D* values of 31 min at 50°C (46), 11.51 at 55°C (47) in turkey, between 2.14 and 8.32 min at 56°C in apple juice (48), and 6.6 min at 55°C (46) and between 0.95 and 4.98 min at 58°C (49) in milk. It has also been observed that cooking temperatures of 53°C or higher result in a rapid decrease in STEC populations during the first hours of cheese making and contribute to the safety of this type of cheese (50). In our study, during ripening, these pathogens remained detectable only after enrichment. Usajewicz and Nalepa (51) observed that at

55°C, *E. coli* cells in milk were inactivated after 180 min. Even if these bacterial strains were stressed and damaged, the authors demonstrated that under optimum conditions (37°C) they were able to repair the damage, whereas storage at 7°C inhibited their regeneration and growth. In the CC scheme, the cheeses are ripened at 9°C, which could explain the inability of surviving STEC to grow after the cooking step.

Factors enabling STEC rates to increase. An increase in the levels of STEC was observed in BTC and the two UPCs. During the first hours of cheese making, the increase in numbers is due to the cell concentration during curd formation, which generally increases the STEC concentration by a factor of 5 for blue-type cheese or nearly 10 for the other cheese types (25, 29; our unpublished data). This increase can occur as a result of bacterial cell entrapment in the curd and contraction after whey expulsion. However, the rate of increase in STEC for the BTC and the two UPCs (approximately 0.5 to 2.5 $\log_{10}$ CFU · g^{-1}, depending on the cheese-making scheme and serotype) clearly indicates bacterial growth. Schlesser et al. (24) have also observed a 1- to 2-$\log_{10}$-CFU · g^{-1} increase in an artificially inoculated cocktail of *E. coli* O157:H7 during cheddar cheese-making operations. At the initial stages of preparation of the BTC and the two UPCs, the temperature (>30°C) is ideal for STEC growth and the moderate acidification can only slow down this growth. Indeed, the pH did not fall below 5 in the BTC and the two UPCs during the first 24 h of cheese making. It has been demonstrated that *E. coli* O157:H7 can grow at a pH of 4.5 to 9 *in vitro* (43).

Factors allowing a decrease in STEC levels during ripening. The length of the ripening has an impact on STEC survival: during this step, a_w decreased (45). The minimum a_w for *E. coli* growth is 0.95 (52), and Glass et al. (53) observed that an a_w of 0.92 in cheese slices was sufficiently low to allow a decrease of STEC strains. In BTC, the a_w during the ripening was lower than the a_w observed for the other cheese-making schemes and fell below 0.90 at day 240. Moreover, cheeses were kept at −2°C between days 28 and 90. Negative temperatures combined with the decrease of a_w and acidic pH may reduce STEC populations even if STEC can survive in foods stored at refrigeration temperatures (54). For UPC1-LR, the decrease in a_w during ripening may also contribute to the decrease in STEC level after day 90. Interestingly, a_w decreased faster in the rind than in the core, but the pH remained constant at about 7.7 in the rind. This could explain the lower rate of *E. coli* O26:H11 in the rind than in the core at day 240. For BTC and UPC1-LR, viable strains were still isolated (after enrichment or not) at day 240. Our results are in agreement with those of Schlesser et al. (24), who have shown that populations of *E. coli* O157:H7 in cheese aged for 60 and 120 days at 7°C decreased less than 1 and 2 $\log_{10}$ CFU · g^{-1}, respectively. Similarly, D'Amico et al. (23) observed that three strains of *E. coli* O157:H7 remained detectable after enrichment for more than 270 days in artificially inoculated cheddar and Gouda cheeses. These results are in agreement with those of others studies (23, 24) and confirm that the 60-day aging is effective for reducing all the STEC strains, as was observed for BTC, UPC1-LR, and CC, but cannot eliminate all the STEC cells.

Conversely, a short ripening step cannot achieve a significant reduction in a_w. This is the case for UPC2-SR. With this cheese, no decrease in STEC level was observed after the first stages of cheese making. This high stationary level has been previously described. Ramsaran et al. (55) observed that *E. coli* O157:H7 in cow's milk inoculated at 4 $\log_{10}$ CFU · ml^{-1} survived at more than 6 $\log_{10}$ CFU · g^{-1} at the end of the storage period (75 days).

The survival of STEC during ripening and refrigerated storage of raw-milk cheese products may be affected by a combination of factors (time, pH, a_w, temperature). Indeed, multiple hurdles are often found to outperform a single hurdle (56).

Serotype effect. Statistically, no strain effect for the same serotype was observed in LC or UPC2-SR. However, we must bear in mind that only a few different strains were tested for each serotype. Similarly, D'Amico et al. (23) have shown no significant strain effect for the three *E. coli* O157:H7 strains employed in artificially inoculated cheddar cheese. Interestingly, a serotype effect was observed in BTC and the two UPCs, with less growth of the serotype O157:H7 strains than that of the serotype O26:H11, O103:H2, and O145:H28 strains being observed.

We can hypothesize that serotypes O26:H11, O103:H2, and O145:H28 may be better competitors than serotype O157:H7. *E. coli* O157:H7 is known to have lost its sorbitol fermentation and β-glucuronidase activity during its evolution (57). Other mutations may affect its behavior in dairy products. Furthermore, the *stx* genes are carried on lambdoid phages, and stresses are known to induce the lytic cycle of this prophage (58, 59). Perhaps if serotype O157:H7 is more sensitive than the other serotypes during cheese making, activation of the lambdoid prophage lytic cycle could reduce the *E. coli* O157:H7 population in the cheese. It will be necessary to test other *E. coli* O157:H7 strains from different origins or the particular sorbitol-fermenting *E. coli* O157:NM nonmotile (NM) strain.

In conclusion, this study has demonstrated that a heating step and sudden, rapid acidification allow efficient STEC removal for CC and LC. Conversely, moderate acidification and moderate temperatures allow these bacteria to grow in BTC and the two UPCs. In this case, STEC levels can be reduced by a long ripening step.

With BTC and UPC1-LR, most strains remained detectable after enrichment even after several months of ripening. However, one must bear in mind that the STEC strains were inoculated at high concentrations that were higher than those found in naturally contaminated milk. STEC levels at the end of ripening are lower in artisanal or industrial cheeses than they were in our experimental cheeses.

These data provide an interesting basis for assessing the final concentration in cheese under more realistic raw-milk contamination conditions, using a predictive microbiology and quantitative exposure assessment approach (60). It will be important to confirm the absence of a strain effect, as only a few strains of each serotype were tested here. Moreover, if the weak growth of *E. coli* O157:H7 is confirmed, it will be interesting to study the physiological state of this STEC serotype during cheese manufacture (stress, induction of lambdoid prophage, etc.). It will also be interesting to study the impact of the autochthonous milk microflora as well as starter lactic acid bacteria and molds on the behavior of STEC: Duffy et al. described these microorganisms to play an antagonistic role against STEC (61). Other authors, in contrast, have shown that *E. coli* growth was not affected by these bacteria (62) and that *Penicillium roqueforti* can enhance the growth and survival of *E. coli* O157:H7 *in vitro* (63). Future experiments will be necessary to understand the mechanisms involved in STEC inhibition during the making and ripening of cheeses.

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